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Cholesterol Accumulation in NPC1-Deficient Neurons Is Ganglioside Dependent

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Summary

Niemann-Pick type C (NPC) disease is a lysosomal disorder commonly caused by a recessive mutation in *NPC1*, which encodes an integral membrane protein with regions of homology to the morphogen receptor, Patched, and to 3-hydroxy-3-methylglutaryl coenzyme A reductase [1, 2]. Neurons in NPC disease exhibit extensive storage of free cholesterol and glycosphingolipids (GSLs), including GM2 and GM3 gangliosides [3, 4, 5]. Most studies have viewed cholesterol storage as primary, with NPC1 functioning as a retroendocytic transporter for regulation of cholesterol homeostasis [3, 6, 7, 8]. Here, we analyze the effects of genetically depriving NPC neurons of complex gangliosides by creating mice doubly deficient in both NPC1 and the GSL synthetic enzyme, GM2/GD2 synthase (GalNAcT). Ganglioside and cholesterol expression in neurons of NPC1$^{-/-}$/GalNAcT$^{+/+}$, NPC1$^{-/-}$/GalNAcT$^{-/-}$, NPC1$^{+/+}$/GalNAcT$^{-/-}$, and WT mice was examined in situ by immunocytochemical and histochemical methods. Neurons in double-deficient mice lacked intraneuronal GM2 accumulation as expected, but remarkably also exhibited absence or dramatic reduction in free cholesterol. Neurons storing cholesterol consistently showed GM3 accumulation but some GM3-positive neurons lacked cholesterol storage. These findings provide a compelling argument that cholesterol sequestration in NPC1-deficient neurons is ganglioside dependent and suggest that the function of NPC1 in these cells may be more closely linked to homeostatic control of GSLs than cholesterol.

Results and Discussion

One approach to understanding the cellular mechanisms underlying lipid storage in NPC disease, and therefore the function of NPC1, is through examination of the effects of pharmacologic or genetic reduction of specific classes of accumulating materials. Cholesterol-lowering drugs like lovastatin and cholestyramine in humans [9] or probucol and nifedipine in mice [10] successfully decreased circulating plasma cholesterol levels but did not impact brain cholesterol levels or the clinical course of disease. In addition, low-density lipoprotein receptor-deficient NPC1$^{-/-}$ mice displayed unaltered features of neurodegeneration [11]. These studies suggest that cholesterol depletion alone is not sufficient to rescue the NPC cellular or clinical phenotype. In contrast, treatment of NPC1-deficient mice and cats with *N*-butyldeoxynorjirimycin (*N*B-DNJ), a pharmacological inhibitor of GSL biosynthesis, not only decreased GSL accumulation in brain but also successfully ameliorated the clinical disease phenotype and, in NPC1$^{-/-}$ mice, increased longevity [12].

Expression of individual types of gangliosides can also be reduced genetically in NPC1$^{-/-}$ mice by crossing these animals with mice in which specific genes for GSL synthesis (e.g., GalNAcT) have been deleted. In a previous study, such NPC1$^{-/-}$ mice lacking GalNAcT and thus GM2 and other complex gangliosides were reported to exhibit no improvement in the clinical course of disease, suggestive of a lack of significance of gangliosides in NPC1 disease pathogenesis [13]. Given the potential importance of these findings, we performed a more extensive analysis of the relationship between ganglioside and cholesterol storage in different brain regions of mice lacking both NPC1 and GalNAcT.

Brains from a total of 18 mice with the following genotypes, 5 NPC1$^{-/-}$/GalNAcT$^{-/-}$, 3 NPC1$^{+/+}$/GalNAcT$^{-/-}$, 5 NPC1$^{-/-}$/GalNAcT$^{+/+}$, and 5 NPC1$^{+/+}$/GalNAcT$^{+/+}$ at 6 and 9 weeks of age, were analyzed for the presence of unesterified cholesterol using filipin staining. In comparison to control mice carrying wild-type GalNAcT and NPC1, where unesterified cholesterol was not accumulated, NPC1$^{-/-}$ mice exhibited pronounced unesterified cholesterol sequestration within neurons in all cortical areas, consistent with an earlier report [5]. Filipin staining was principally concentrated in abnormal axon hillock/perikaryal swellings (meganeurites) characteristic of neuronal storage diseases [14]. Storage was particularly exacerbated in large layer V cortical pyramidal neurons (Figure 1A, upper middle panel) and in neurons throughout the hippocampus, as exemplified by the dentate gyrus and CA4 pyramids (lower middle panel). Introduction of the GalNAcT KO phenotype to NPC1-deficient animals (Figure 1A, right panels) caused unesterified cholesterol accumulation to be markedly decreased throughout the cerebral cortex, the hippocampus, and other subcortical regions. Filipin staining in GalNAcT knockout mice with functional NPC1 resembled that observed in wild-type mice in that there was no accumulation of filipin-positive material within neuronal perikarya (not shown).

Although substantial reduction in cholesterol accumulation was evident throughout the brain in double-deficient animals, careful analysis did reveal the presence of cholesterol storage in subsets of neurons in many brain regions including cerebral cortex. This result is in contrast to the report mentioned above that detected no filipin labeling in cerebral neurons of genetically equivalent mice [13]. A likely explanation for this

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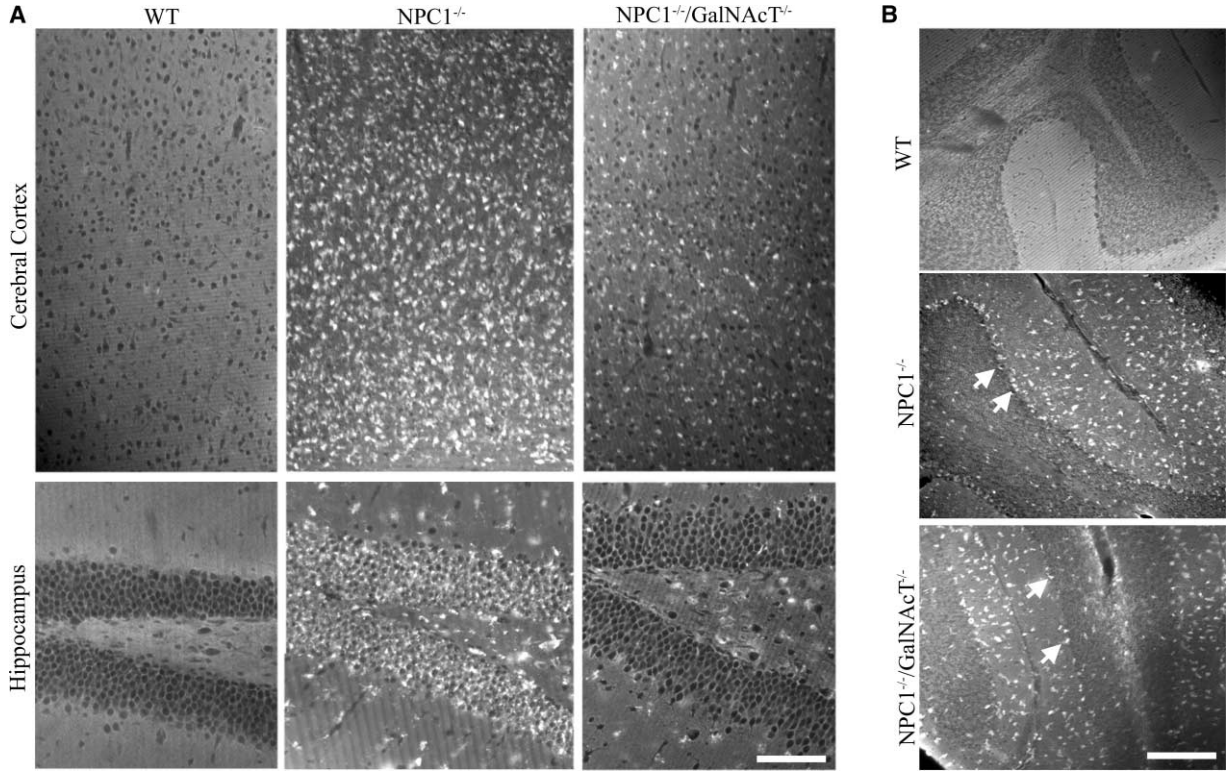

Figure 1. Cholesterol Expression Was Analyzed in the Cerebrum and Cerebellum of NPC1$^{-/-}$ Mice Lacking Complex Gangliosides

Mice heterozygous for the NPC1 mutation and the GalNAcT deletion were crossed to generate double mutant NPC1$^{-/-}$ mice unable to synthesize higher order gangliosides.

(A) Filipin was used to stain for unesterified cholesterol (white) in cerebral cortex (top panel) and hippocampus (bottom panel). Cholesterol was not accumulated in normal wild-type (WT) brains (left panel), but was stored throughout neurons of NPC-affected brains (middle) and to a considerably lesser extent in double-deficient mouse brains (right panel).

(B) Analysis of unesterified cholesterol in cerebella of NPC1$^{-/-}$ (middle panel) and double-deficient animals (bottom panel) showed no difference in distribution as both exhibited numerous filipin-positive profiles throughout the cerebellar cortex, and particularly in the molecular layer. Arrows illustrate Purkinje neurons that are still present and laden with unesterified cholesterol. As expected, WT mice (top panel) do not abnormally sequester unesterified cholesterol within cell bodies. Bar = 120 μm for upper panels of (A) and 80 μm for lower panels of (A); Bar in (B) = 200 μm.

difference is that the portion of the cerebrum analyzed in the previous study may have represented a particular subcortical region in which cholesterol accumulation was absent or below detection.

In the cerebellum (Figure 1B), a severely affected brain region in NPC1 disease that exhibits extensive Purkinje cell death and marked gliosis [5], the limitation of complex ganglioside synthesis in double-deficient mice did not significantly alter the degree of cerebellar degeneration. Staining with calbindin, a selective Purkinje cell marker, at 9 weeks of age revealed that both NPC1$^{-/-}$ and double-deficient mice had few of these neurons surviving (not shown). Filipin staining of cerebellum at this age revealed the presence of numerous presumptive microglia/macrophages in the molecular layer of both types of animals (Figure 1B, middle and lower panels), with this being a characteristic index of the absence of Purkinje cells in a given area. Purkinje cells that were present in both NPC1$^{-/-}$ and double-deficient mice at this age showed substantial perikaryal accumulation of cholesterol (arrows).

By immunocytochemistry, GM3 ganglioside expression was not detected in normal brain but was found significantly elevated in NPC1-deficient neurons, as previously reported [5], and similar levels of GM3 staining were evident in double-deficient animals (Figure 2A). The latter finding of no significant compensatory increase in GM3 storage in double-deficient mice versus NPC1$^{-/-}$ animals is consistent with HPTLC analysis of this model in earlier studies [13] and suggests that the accumulation of GM2 and GM3 gangliosides in NPC1 disease may be occurring by independent mechanisms. Comparison of GM3 immunolabeling and filipin histochemical staining in several brain regions of double-deficient mice indicated that the unesterified cholesterol pattern paralleled that of GM3 ganglioside storage (Figures 2B–2D). For example, cholesterol often accumulated in large neurons of the deeper layers of the cerebral cortex and in the CA3 region of hippocampus (right panels of Figures 2B and 2D, respectively), with this occurring in a manner similar to GM3 storage in these same areas (left panels of Figures 2B and 2D). In CA1 hippocampal neurons where GM3 storage was absent, cholesterol accumulation likewise did not occur (Figure 2C). Thus, although unesterified cholesterol was dramatically decreased in most areas of brain in the double-deficient

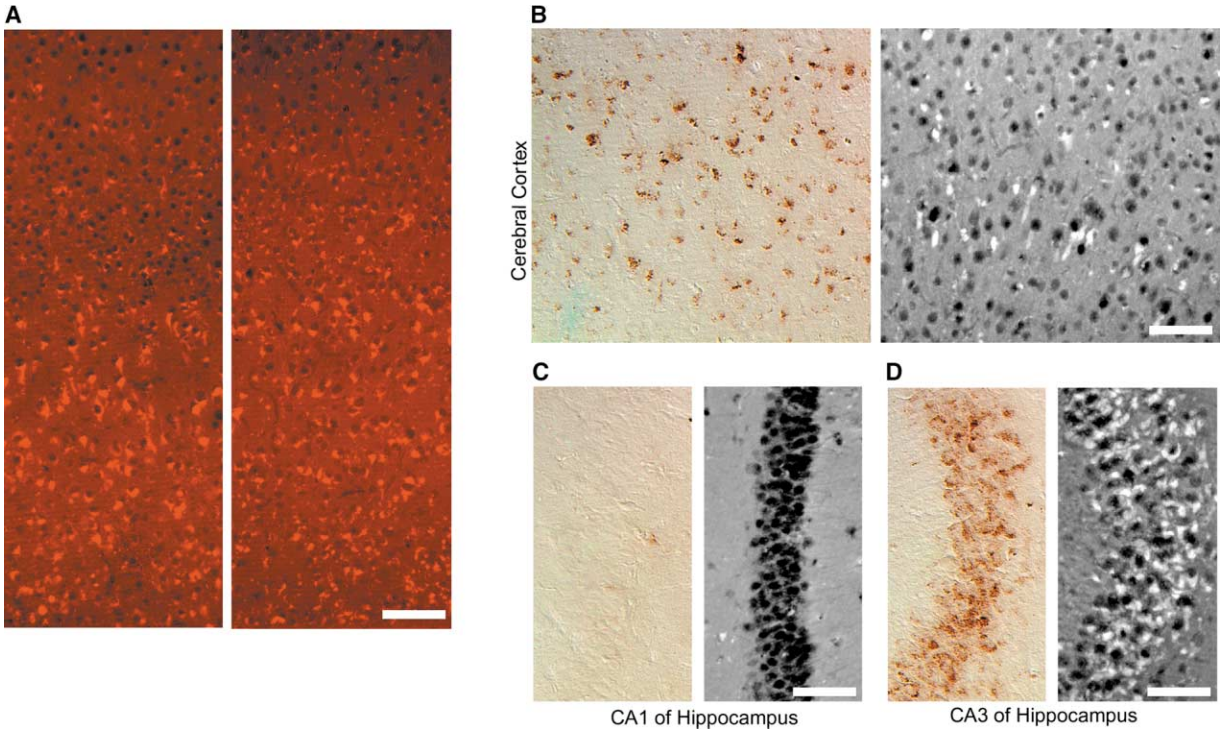

Figure 2. Regional Expression of GM3 Ganglioside and Unesterified Cholesterol Were Compared in Cerebral Cortex and Hippocampus

Analysis of the expression of the simple ganglioside, GM3, in NPC$^{-/-}$ (A, left) and double-deficient mouse cerebral cortex (A, right) showed no obvious compensatory increase in GM3 expression when GalNAcT was absent. The expression of GM3 ganglioside (brown) was assessed in situ by immunocytochemistry and compared to unesterified cholesterol (white) determined by filipin staining in different brain regions. In cortex (B) as well as in the CA1 (C) and CA3 (D) regions of the hippocampus, unesterified cholesterol expression (right panels) was proportional to ganglioside expression (left panels). Notably, in CA1 neurons where little to no GM3 accumulation was found, cholesterol was also absent; in CA3 neurons where GM3 was abundant, cholesterol accumulation was conspicuous. Bar = 70 μm for (A); 63 μm for (B), (C), and (D).

animals, when it did occur, its accumulation consistently followed that of GM3 ganglioside in those regions.

To determine whether the observed regional accumulation of unesterified cholesterol was localized to the same neurons that were accumulating GM3 ganglioside, double staining with GM3 antibody and filipin was performed. Results showed that GM3 and unesterified cholesterol were commonly localized to the same neurons within the cerebral cortex (Figure 3), a finding also observed in the CA3 region of the hippocampus (not shown). Occasionally, cholesterol storage was absent in neurons storing GM3 (Figure 3, inset A, arrowheads), an indication that GM3 accumulation was not cholesterol dependent. Interestingly, neurons lacking GM3 storage typically showed significant immunoreactivity for GD3, the other ganglioside made in the absence of GalNAcT but not one reported to be pathologically elevated in NPC1 disease [5, 15]. The presence of cholesterol storage in neurons containing GM3 but not in those with GD3 alone suggests not only linkage of the cholesterol storage mechanism with GM3 accumulation but also that different types of neurons may have different capacities and/or mechanisms for processing these glycolipids. Normal neurons, which do not exhibit detectable free cholesterol with filipin labeling [5], are also known to express ample amounts of GD3 ganglioside immunoreactivity in a punctate cytoplasmic pattern, consistent with GD3 being a major ganglioside of neurons in mammalian brain [16].

One brain region of double-deficient mice in which cholesterol accumulation was not significantly diminished was the cerebellum (Figure 1B). When younger double-deficient mice were analyzed at 5 and 6 weeks, the majority of Purkinje cells were intact and showed punctate GM3 ganglioside colocalized to the same neurons exhibiting filipin-positive vesicles (Figures 4A and 4B). In contrast, littermate mice recessive for the NPC1 defect but normal for GalNAcT analyzed under the same conditions, while positive for GM2 and cholesterol accumulation, did not show detectable levels of GM3 storage (Figure 4C). The presence of GM3 in Purkinje cell somata of younger double-deficient animals and its association with cholesterol storage indicates that in the absence of other gangliosides, altered expression of GM3 may be elicited, at least in this cell type. This occurrence of GM3 and its colocalization with cholesterol is consistent with findings from other brain regions and with the suggestion that cholesterol accumulation in double-deficient neurons is GM3 dependent. However, the degree of GM3 accumulation relative to cholesterol was less than that typically observed elsewhere, e.g., in cerebral cortical neurons where the two materials were equally conspicuous (Figure 3).

Average weight, longevity, and onset of clinical disease in NPC1$^{-/-}$ and in double-deficient mice were also investigated. In addition to the five double-deficient mice described above, an additional six of these animals were produced and maintained for longevity studies.

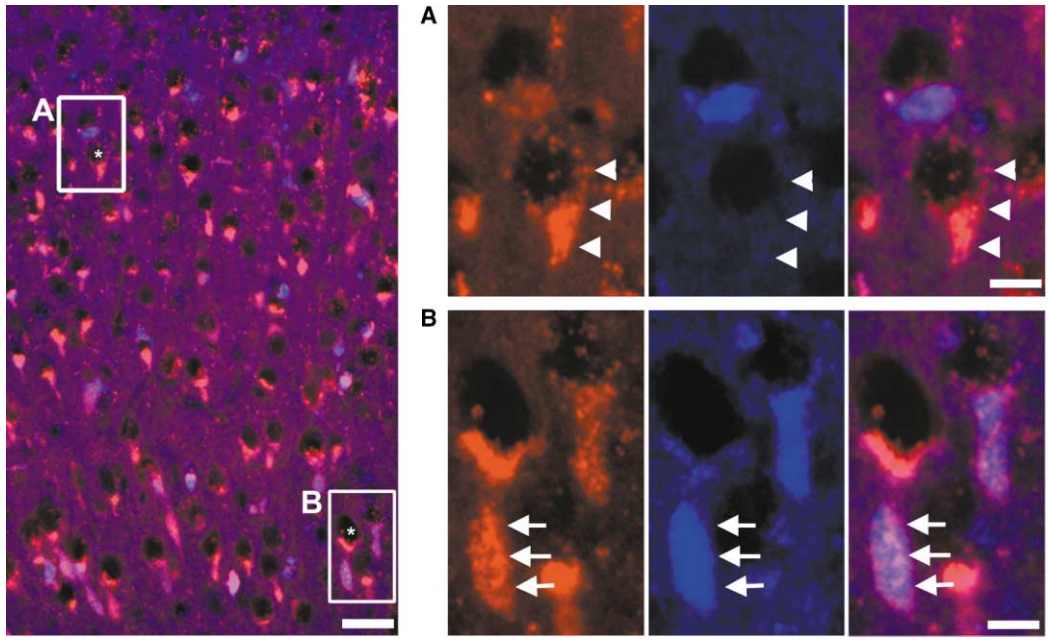

Figure 3. Coexpression of Cholesterol and GM3 Ganglioside in Layer V Pyramidal Neurons of Double-Deficient Mouse Cerebral Cortex

Double labeling with an antibody to GM3 (red) and filipin histochemistry (blue) was performed on 15 μm sections of double-deficient mouse cerebral cortex and analyzed by fluorescence microscopy. Examples of neuronal nuclei are demarcated with an asterisk (*). Some neurons exhibiting GM3 storage did not demonstrate cholesterol storage in the same plane (inset A, arrowheads), but cell profiles positive for filipin and lacking GM3 were not observed. Cholesterol storage was localized to the same neurons as GM3 in meganeurites (arrows) and in associated cell bodies of layer V pyramidal neurons (inset B). Bar = 60 μm for left panel; 20 μm for insets A and B.

Similar to published assessments [13], we found that the NPC1$^{-/-}$ clinical phenotype was not consistently improved by genetic depletion of complex gangliosides. Double-deficient animals exhibited clinical features typical of NPC1$^{-/-}$ disease and some exhibited this phenotype earlier than littermate NPC1$^{-/-}$ mice. Double-deficient animals typically remained smaller than NPC1$^{-/-}$ mice throughout life and some developed ataxia, tremor, and decreased mobility by approximately 5 weeks of age, whereas NPC1$^{-/-}$ mice in our colony consistently

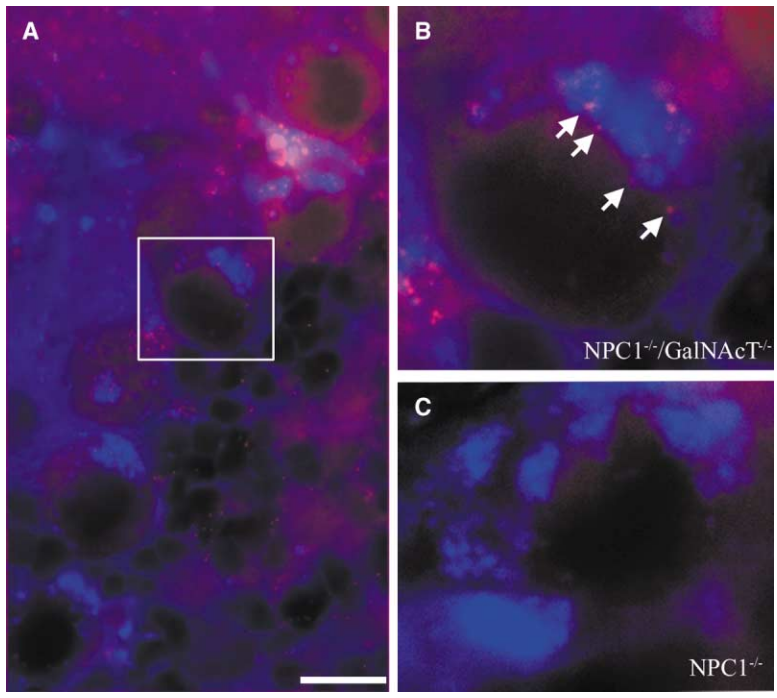

Figure 4. Cholesterol and GM3 Ganglioside Coexpression Was Analyzed in Purkinje Neurons of Double-Deficient Mice versus NPC1$^{-/-}$ Mice

Double labeling with antibody to GM3 (red) and filipin (blue) was performed on 15 μm sections of cerebellum and analyzed by fluorescence microscopy. Cholesterol storage was abundant in Purkinje cells of 5-week-old double-deficient (A, B inset) and in NPC1$^{-/-}$ mice (C). Purkinje cells in double-deficient animals also exhibited GM3 accumulation in association with filipin-reactive regions of the perikarya (B, arrows) whereas age-matched NPC1$^{-/-}$ Purkinje cells exhibited no GM3 immunoreactivity in conjunction with cholesterol storage (C). Bar = 13 μm and applies to (A).

did not develop equivalent symptoms until 6–7 weeks. However, when evaluated for longevity, a wide range of life spans was detected in the double-deficient mice, from 6 to 15 weeks, compared to 10–11 weeks for NPC1$^{-/-}$ mice housed under similar conditions. Notably, the three double-deficient animals that lived for 15 weeks were housed in individual cages and had ready access to powdered food. This difference in husbandry practice may explain the difference in our findings compared to the earlier published report on this double mutation [13]. This extended survival time, interestingly, is about equivalent to that observed when NPC1$^{-/-}$ mice were administered GSL synthesis inhibitors [12, M.C.G.-L. and S.U.W., unpublished data].

In summary, the massive accumulation of GM2 and GM3 gangliosides along with neutral glycosphingolipids in NPC1$^{-/-}$ disease and the ability of genetic and pharmacologic inhibition of GSL synthesis to ameliorate disease progression and increase longevity suggest that one function of the NPC1 protein is centered on homeostatic control of GSL expression [5, 12]. Conceivably, NPC1 mediates removal of gangliosides residing at the end of the GSL degradative pathway [17], namely GM2 and GM3, and/or other simple GSLs (lactosylceramide, glucosylceramide) from the late endosomal/lysosomal compartment and shuttles them to the Golgi as part of a retroendocytic recycling system critical for GSL homeostasis, not unlike that originally proposed for cholesterol in NPC disease [3, 7]. It is also possible, however, that neither GSLs nor cholesterol are directly transported by NPC1 since this protein has also been implicated more generally in transport of lipophilic molecules and certain fatty acids [18]. Nonetheless, the current studies indicate that cholesterol sequestration in neurons is not itself a primary event but rather is dependent on the presence of ganglioside accumulation. That is, elimination of complex gangliosides including the pathological storage of GM2 from NPC1$^{-/-}$ brains in the double-deficient model caused dramatic reduction in cholesterol storage. Neurons that did store cholesterol consistently showed the presence of GM3, though the opposite was not true: some neurons that were GM3 positive lacked cholesterol storage. If NPC1 does function to control GSL homeostasis, the heightened presence of GM2 ganglioside in NPC1$^{-/-}$ brain may be indicative of this ganglioside exiting the Golgi/TGN in exocytic vesicles similar to that believed to occur in normal, developing neurons [19]. A relative increase in synthesis of GM2 and possibly other GSLs would explain why GM2 ganglioside is so abundant in NPC1-deficient neurons in spite of the presence of normal catabolic machinery for its degradation, and likewise, is consistent with the finding that GSL synthesis inhibitors slow disease progression. To better understand this process, it will be important to explore further the precise vesicular location of individual GSLs as they accumulate in this and related storage diseases.

Supplemental Data

Supplemental Experimental Procedures are available with this article online at http://www.current-biology.com/cgi/content/full/13/15/1324/DC1.

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